A novel β1,3-*N*-acetylglucosaminyltransferase (β3Gn-T8), which synthesizes poly-*N*-acetyllactosamine, is dramatically upregulated in colon cancer☆,☆☆

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Abstract A new member of the UDP-*N*-acetylglucosamine: β-galactose β1,3-*N*-acetylglucosaminyltransferase (β3Gn-T) family having the β3-glycosyltransferase motifs was identified using an in silico method. This novel β3Gn-T was cloned from a human colon cancer cell line and named β3Gn-T8 based on its position in a phylogenetic tree and enzymatic activity. β3Gn-T8 transfers GlcNAc to the non-reducing terminus of the Galβ1–4GlcNAc of tetraantennary *N*-glycan in vitro. HCT15 cells transfected with β3Gn-T8 cDNA showed an increase in reactivity to both LEA and PHA-L4 in a flow cytometric analysis. These results indicated that β3Gn-T8 is involved in the biosynthesis of poly-*N*-acetyllactosamine chains on tetraantennary (β1,6-branched) *N*-glycan. In most of the colorectal cancer tissues examined, the level of *β3Gn-T8* transcript was significantly higher than in normal tissue. β3Gn-T8 could be an enzyme involved in the synthesis of poly-*N*-acetyllactosamine on β1–6 branched *N*-glycans in colon cancer.

Keywords: Glycosyltransferase; β1,3-*N*-Acetylglucosaminyltransferase; Polylactosamine; Colon cancer; *N*-Glycan

1. Introduction

The complex carbohydrate chains of glycoproteins (*O*-glycans and *N*-glycans), glycolipids, and proteoglycans represent secondary gene products formed through the reactions of many glycosyltransferases. In the past few years, rapid advances in cDNA cloning achieved utilizing databases for genome and expressed sequence tag (EST) projects of human, mouse, *Caenorhabditis elegans*, and Drosophila have led to the discovery of a large number of novel glycosyltransferase genes. We also have cloned and analyzed many human glycosyltransferase genes with the aid of bioinformatics technology.

Glycosyltransferases can be grouped into functional subfamilies based on similarities of sequence, which reflect their enzymatic character; donor specificity, acceptor specificity, and specific linkage between donor and acceptor. Three β3-glycosyltransferase (β3-GT) motifs, XIRX(S/T)W(G/L/M), (F/Y)-XXXXDXD and (E/D)DVXXGX, which we found in a previous study, are commonly encoded in β3-GTs that combine two sugars with a β1,3-linkage [1–4]. These specific motifs are shared by β3Gal-Ts, β3GalNAc-Ts and β3Gn-Ts. It was suggested that these similarities may in fact reflect a structural component for catalysis of the β-1,3-linkage. In the past, we succeeded in the molecular cloning of a series of β3-GT genes. Thirteen human β3-GTs, i.e., five β3Gal-Ts, two β3GalNAc-Ts and six β3Gn-Ts, have been identified and reported [1–8].

Poly-*N*-acetyllactosamine (Polylactosamine) is a unique glycan comprising *N*-acetyllactosamine (LacNAc) repeats, (Galβ1–4GlcNAcβ1–3)n, and attached to *O*-glycans, *N*-glycans and glycolipids. Transfection experiments and in vitro enzymatic analysis have demonstrated that each of β3Gn-T2, -T3, -T4, -T5, and -T7 is able to catalyze the initiation and

☆ The nucleotide sequence data reported in this paper will appear in the DDBJ/GenBank Data Bank with the accession number AB175895 for human β3Gn-T8.

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Abbreviations: β3Gn-T, UDP-GlcNAc: β-galactose β1,3-*N*-acetylglucosaminyltransferase; β3Gal-T, UDP-galactose: β-*N*-acetylglucosamine β1,3-galactosyltransferase; β3GalNAc-T, UDP-GalNAc: β-galactose β1,3-*N*-acetylgalactosaminyltransferase; UDP-GlcNAc, uridine diphosphate-*N*-acetylglucosamine; PBS, phosphate-buffered saline; bp, base pair(s); PCR, polymerase chain reaction; FBS, fetal bovine serum; PA, pyridylaminated

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elongation of polylactosamine chains, however they exhibit a different substrate specificity dependent on the length of the polylactosamine chain [1,3]. Our in vitro analysis previously demonstrated that β3Gn-T2 is most active toward polylactosamine substrates having 2–5 LacNAc repeats [1]. Polylactosamine chains are often modified to express differentiation antigens and functional oligosaccharides. Yeh et al. [9] reported that the extension of core 1 mucin-type *O*-glycan is directed by β3Gn-T3 through addition of GlcNAc, resulting in the synthesis of the "GlcNAcβ1–3Galβ1–3GalNAc-*O*-Ser/Thr" structure. This "core 1-extended *O*-glycan" often forms the 6-sulfo sialyl Lewis x antigen, the epitope recognized by the monoclonal antibody MECA-79, on the terminal structure. This epitope is known to be expressed in high endothelial venules (HEV) and function as an L-selectin ligand required for lymphocyte homing. β3Gn-T5 exhibited the strongest activity to transfer GlcNAc to glycolipid substrates, such as lactosylceramide (LacCer) and neolactotetraosylceramide (nLc_4Cer; paragloboside), resulting in the synthesis of Lc_3Cer and neolactopentaosylceramide (nLc_5Cer). β3Gn-T6 showed β3Gn-T activity toward GalNAcα-pNp and GalNAcα1-serine/threonine. β3Gn-T6 effectively transferred GlcNAc to the GalNAc residue on mucins, resulting in the synthesis of a core 3 structure. It is known that polylactosamine chains are elongated on core 3 and/or core 4 structures [10,11]. Seko and Yamashita [8] demonstrated that β3Gn-T7 can act on keratan sulfate (KS)-related oligosaccharides, for example, Galβ1–4(SO_3^--6)GlcNAcβ1–3Galβ1–4(SO_3^--6)GlcNAc. We demonstrated that β3Gn-T7 is also involved in the synthesis of polylactosamine chains using HCT15 cells stably transfected with the *β3Gn-T7* gene (unpublished data). Thus, all β3Gn-Ts, β3Gn-T2, -T3, -T4, -T5, and -T7, except for -T6, can transfer GlcNAc to Gal with a β1,3-linkage to synthesize a polylactosamine chain. However, each differs in its preference for acceptor molecules, i.e., core 1 *O*-glycan, glycolipids or KS. Each enzyme may have distinct roles in physiological processes.

Polylactosamine is often modified to carry important carbohydrate structures such as Lewis-related antigens [12–15], HNK-1 antigen [16], etc., and has many major roles in physiological functions.

In cancer metastasis, polylactosamine and related structures play important roles in cell–cell interaction, in cell-extracellular matrix (ECM) interaction [17], in immune response [18] and in determining metastatic capacity [19]. Tri/tetra-antennary (β1,6-branched) *N*-glycans containing polylactosamine on a β1,6-branch act on a variety of malignant phenotypes of tumor cells, affecting cell proliferation [20] and metastatic potential [21–26].

In this study, we cloned a novel β3Gn-T, named β3Gn-T8, with the aid of bioinformatics. This enzyme was identified to be a polylactosamine synthase and acted on tetraantennary *N*-glycans having a β1–6 branch.

2. Materials and methods

2.1. Construction and purification of human β3Gn-T8 proteins fused with FLAG peptide

We performed a BLAST search of the EST databases and identified a cDNA (AW444713), homologous in amino acid sequence to the open reading frame (ORF) of β3Gn-T3 [3]. On searching the human genomic DNA database, we found a single contig (AC011462) containing the EST sequence. The putative catalytic domain of the enzyme (amino acids 76–1194) was amplified by PCR from Colo205 cDNA as a template and two primers, 5′-GCCAAGCTTACATCCGAGTCCCGGCTCAG-3′ and 5′-CGGAATTCTCAGCACTGGAGCCTTGGGT-3′. The amplified fragment was digested with the restriction endonucleases *Hin*dIII and *Eco*RI, then inserted into pFLAG-CMV3 (Sigma, MO) to construct pFLAG-CMV3-β3Gn-T8. The putative catalytic domain of β3Gn-T8 was expressed as a secreted protein fused with a FLAG peptide in 293T cells (a human embryonic renal cancer cell line). A 12-ml volume of culture medium was mixed with anti-FLAG M1 antibody resin (Sigma, MO). The protein–resin mixture was washed twice with 50 mM TBS (50 nM Tris–HCl, pH 7.4, and 150 mM NaCl) containing 1 mM $CaCl_2$ and suspended in 100 μl of each of the assay buffers.

2.2. Substrate specificity of β3Gn-T8

To determine a donor and acceptor substrate for β3Gn-T8, all combinations of nucleotide sugars and monosaccharides were screened by a method described previously [4].

The basic reaction mixture for assaying Gn-T activity contained 14 mM HEPES buffer, pH 7.4, an appropriate concentration of UDP-GlcNAc, 10 mM $MnCl_2$, 0.15% Triton CF-54, 0.75 mM ATP, a suitable amount of acceptor substrate, and the purified enzyme. After incubation at 37 °C for 16 h, the product was analyzed using various techniques as described below.

A Gn-T assay of human β3Gn-T8 using the oligosaccharides was performed as follows: 50 μM UDP-GlcNAc (Sigma), 4.5 μM (50 nCi) [^{14}C]UDP-GlcNAc (Amersham Biosciences, Amersham Place, UK) and 10 μM of acceptor substrate were added to 20 μl of the basic reaction mixture. The acceptor substrate used in this study, listed in Table 1, was purchased from Calbiochem (La Jolla, CA), Sigma and Toronto Research Chemicals Inc. (Ontario, Canada). As summarized in Table 2, the Gn-T assay for *N*-glycans was performed using pyridylaminated substrates: 2 μM of pyridylaminated acceptor substrate (TaKaRa, Japan) and 50 μM UDP-GlcNAc (Sigma) were added to the basic reaction mixture. The fluorescence intensity of the products was determined by HPLC on a PALPAK type R column (4.6 × 250 mm; TaKaRa) according to the instruction manual. Parallel reactions as controls were done in the absence of UDP-GlcNAc to identify the products.

2.3. N-acetylglucosaminyltransferase assay with glycoproteins

The transfer of GlcNAc by β3Gn-T8 to glycoproteins was performed in 20 μl of a basic reaction mixture, containing 50 μM UDP-GlcNAc, 4.5 μM (50 nCi) UDP-[^{14}C]GlcNAc, and 40 μg of α1-acid glycoprotein (Sigma) or ovomucoid (Sigma). After incubation at 37 °C for 16 h, the enzyme reaction was terminated by treatment at 100 °C for 3 min, then 2.5 μl of reaction mixture was treated with H_2O or Glycopeptidase F (GPF, TaKaRa, Ohtsu, Japan) under denaturing conditions according to the instruction manual, and subjected to 10% SDS–PAGE. The radioactive intensities of the bands obtained were measured with a FLA-3000 Imaging Analyzer (Fujifilm, Tokyo, Japan).

Table 1
Substrate specificity of recombinant β3Gn-Ts for mono-, di-saccharide and core structures of *O*-glycans

Acceptor substrate	β3Gn-T8	β3Gn-T2	β3Gn-T7
Galβ1-4GlcNAc-α-pNp	100	4592.2	100
Lactoside-α-Bz	27	1621.4	27.1
GalNAc-α-pNp	ND	8.6	ND
Gal-α-pNp	ND	16.5	0.8
Gal-β-oNp	ND	24.2	2.6
Fuc-α-pNp, GalNAc-α-Bz, GlcNAc-α-Bz, GlcNAc-β-Bz, Glc-α-pNp, Glc-β-pNp, Xyl-α-pNp, Xyl-β-oNp, Man-α-Bz, ManNAc-α-Bz, core 1-pNp, core 2-pNp, core 3-pNp, core 6-pNp	ND	NT	ND

NT: not tested, ND: not detected.

Table 2
Substrate specificity of recombinant β3Gn-Ts for *N*-glycans

Substrate (PA-sugar)	Activity (%)		
	β3Gn-T8	β3Gn-T2	β3Gn-T7
PA-001 Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4GlcNAcβ1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc-PA	ND	79.5	15.1
PA-002 Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4GlcNAcβ1-4 Galβ1-4GlcNAcβ1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc-PA	ND	90.2	21.4
PA-004 Galβ1-4GlcNAcβ1-6 Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4GlcNAcβ1-4 Galβ1-4GlcNAcβ1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc-PA	14.7	100	16.7
PA-009 Galβ1-4GlcNAcβ1-2Manα1-6 Fucα1-6 Galβ1-4GlcNAcβ1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc-PA	ND	73.2	17
PA-010 Galβ1-4GlcNAcβ1-2Manα1-6 Fucα1-6 Galβ1-4GlcNAcβ1-4 Galβ1-4GlcNAcβ1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc-PA	ND	88.4	14.3
PA-011 Galβ1-4GlcNAcβ1-6 Galβ1-4GlcNAcβ1-2Manα1-6 Fucα1-6 Galβ1-4GlcNAcβ1-4 Galβ1-4GlcNAcβ1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc-PA	8.9	99.1	14.9
PA-016 Manα1-6 Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc-PA	ND	ND	ND

2.4. Quantitative analysis of the β3Gn-T8 transcript in human tissues and cell lines by real-time PCR

For quantification of the *β3Gn-T8* transcript, we employed the real time PCR method, as described in detail previously [4]. Total RNA from various human tissues was purchased from Clontech. Normal and cancerous tissues of human colon were obtained from 13 colorectal cancer patients as surgically resected specimens at Fussa Hospital (Tokyo, Japan). The tissues were frozen in liquid nitrogen immediately after surgical resection and stored in liquid nitrogen until RNA extraction. Total cellular RNA was isolated from tissues using an RNeasy® Mini kit (Qiagen, Hilden, Germany). Complementary DNAs were synthesized using oligo(dT)$_{12–18}$ primers and the Superscript First-Strand Synthesis system (Invitrogen). Standard curves for the endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, were generated by serial dilution of pCR2.1 (Invitrogen) DNA containing the *GAPDH* gene. A standard curve for β3Gn-T8 cDNA was generated by serial dilution of pBluescript SKII(−) DNA containing the ORF of *β3Gn-T8*. The primer set and probe for β3Gn-T8 were as follows: forward primer, 5′-GCTGTTGGCCGTCAAGTCAG-3′; reverse primer, 5′-CAGGAAGAGCAGCCGGAT-3′; and probe, 5′-CAGAACGACAGGCCGTGA-3′. Primers, probe, and cDNA were added to the TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), which contained all reagents for PCR. The PCR conditions included 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 10 min, and 50 cycles at 95 °C for 15 s, and 60 °C for 1 min. PCR products were measured continuously with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The relative amount of the transcript was normalized to the amount of *GAPDH* transcript in the same cDNA or the amount of total RNA.

2.5. Construction of vector for expression of β3Gn-T8 in mammalian cultured cells

The fragment of the ORF encoding β3*Gn-T*8 was amplified by PCR from Colo205 cDNA as a template, and two primers, 5′-CTCAAGCTTATGCGCTGCCCCAAGTGCCTTC-3′ and 5′-CTCGAATTCCAGCACTGGAGCCTTGGGTCT-3′. The amplified fragment was digested with the restriction endonucleases *Hin*dIII and *Eco*RI, then inserted into pcDNA3.1(+) (Invitrogen) to construct pcDNA3.1-β3Gn-T8.

2.6. Cell culture and transfection

The tumor cell lines were cultured in RPMI1640 medium (GIBCO BRL, Rockville, MD) supplemented with 10% fetal bovine serum. HCT15 cells, a human colon adenocarcinoma cell line, were transfected with pcDNA3.1-β3Gn-T8 DNA using Lipofectamin 2000 reagent (Invitrogen). At 48 h after the transfection, the cells were subjected to flow cytometric analysis.

2.7. Flow cytometric analysis

For flow cytometry, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated Lycopersicon esculentum agglutinin (LEA; Vector Lab. Inc., CA) and Phaseolus vulgaris-L4 (PHA-L4; Honen, Japan). After incubation, the cells were subjected to flow cytometric analysis with a FACSCalibur (BD Biosciences, CA).

3. Results and discussion

3.1. Determination of nucleotide and amino acid sequence of β3Gn-T8

We obtained a new sequence for the β3-GT family as described under Section 2 and named it β3Gn-T8. An alignment of the amino acid sequence with that of other β3Gn-Ts using ClustalW is shown in Fig. 1. The 1194-bp (397 amino acids) ORF of β3Gn-T8 encoded a typical type II membrane protein, the same as in other β3Gn-Ts, consisting of an *N*-terminal cytoplasmic domain of 4 residues, a transmembane segment of 19 residues, and a stem region and a putative catalytic domain of 374 residues. A comparison of the amino acid sequence with that of other β3-glycosyltransferases revealed that this enzyme lacks the first aspartic acid of the DXD motif, identified as one of the β3Gn-T motifs and a divalent cation-binding site by a crystallization study on other glycosyltransferases [27,28]. Though β3Gn-T8 has an incomplete DXD motif, a divalent cation, Mn^{2+}, is essential for the enzymatic activity like for other members of the family (data not shown). Five cysteine residues were conserved in the seven β3Gn-Ts, which indicate that some of them are essential for maintenance of the tertiary structure of β3Gn-Ts. One possible *N*-glycosylation site was found in the primary sequence, which was conserved in all β3Gn-Ts. The β3Gn-T8 gene was found to be localized to a draft genome sequence (GenBank™ accession No. AC011462) which mapped to 19q13.31 on the human chromosome, 7A3 on the mouse chromosome, and its ORF is composed of a single exon. As shown in the phylogenetic tree (Fig. 2), β3Gn-T8 was categorized into a cluster of the β3Gn-T subfamily. Six members of the β3Gn-T family, including β3Gn-T8, formed a cluster which is separated from other β3-GTs. As mentioned below, we identified that β3Gn-T8 transfers GlcNAc to Gal residue with a β1–3 linkage as do the other β3Gn-Ts. The β3Gn-Ts of the cluster catalyze the synthesis of polylactosamine chains. β3Gn-T5 is positioned on an outer branch away from the other members. β3Gn-T5 acts on a glycolipid, such as lactosylceramide, resulting in the synthesis of lactotoriaosylceramide.

3.2. Determination of glycosyltransferase activity and substrate specificity of β3Gn-T8

FLAG-tagged recombinant β3Gn-T2, -T7 and -T8 were purified from supernatant of 293T cells as described under Section 2. The amounts of each enzyme were made equal for assaying the β3Gn-T activity. Glycosyltransferase activities, Gal-T, Gn-T and GalNAc-T activities, were screened using each donor labeled with ^{14}C. No Gal-T or GalNAc-T activity toward any acceptor substrate was observed (data not shown), whereas Gn-T activity was exhibited toward Gal β1–4 GlcNAc-α-pNp. The optimal pH for the β3Gn-T8 activity was between 7.0 and 7.5 (data not shown). In general, glycosyltransferases having a typical DXD(H) motif require divalent metal ions to exert activity. However, Gn-TV and IGnT, which have no DXD motif, do not require divalent metal ions. β3Gn-T8 has QDD in its amino acid sequence instead of the DDD motif conserved in the other β3Gn-T members. It has been reported that the third aspartic acid in the DXD sequence in β4Gal-T1 binds to divalent metal ions [28]. β3Gn-T8 showed an absolute requirement for divalent metal ions, such as Mn^{2+}, and the optimum concentration of Mn^{2+} was between 2.5 and 10 mM. We examined the effect of other dictations, such as Co^{2+}, Cd^{2+}, Zn^{2+}, Ni^{2+}, and Mg^{2+}, and found that Co^{2+} slightly affected the enzyme activity to ~40% of the level achieved with Mn^{2+} at the same concentration. The other metal ions did not activate the enzyme effectively (data not shown). Substrate specificity for mono-, di-saccharide and core structures of *O*-glycans is summarized in Table 1. The activity of β3Gn-T8 toward Galβ1–4GlcNAc-α-pNp is presented as 100%, and all other activities are given as relative values in Table 1. The activities of β3Gn-T8 toward Galβ1–4GlcNAc-α-pNp and lactoside-α-Bz were almost the same as those of β3Gn-T7, which is responsible for the synthesis of keratan sulfate [8]. But β3Gn-T8 did not show any activity for partial structures of keratan sulfates, such as Galβ1–4(SO_3^--6)GlcNAcβ1–3Galβ1–4(SO_3^--6)GlcNAc (L2L2), Galβ1–4(SO_3^--6)GlcNAcβ1–3(SO_3^--6)Galβ1–4(SO_3^--6)GlcNAc (L2L4) and SO_3^--6Galβ1–4(SO_3^--6)GlcNAcβ1–3(SO_3^--6)Galβ1–4(SO_3^--6)GlcNAc (L4L4). The oligosaccharide substrates having the polylactosamine structures, containing repeats of lactosamine (Galβ1–4GlcNAc; LN), were labeled with 2-aminobenzamide (2AB) and used as acceptor substrates [1,14]. But no activity toward any polylactosamine oligosaccharide was detected in β3Gn-T8 (data not shown).

To determine whether β3Gn-T8 can transfer GlcNAc to glycoproteins, α1-acid glycoprotein and ovomucoid, which, respectively, possess tetraantennary complex type and pentaantennary complex type *N*-glycans, were used as acceptor substrates. As shown in Fig. 3, β3Gn-T8 transferred GlcNAc to these glycoproteins. After digestion of these products with GPF, radioactive signals disappeared. This result indicated that *N*-glycans on these proteins could be acceptor substrates. No activity was detected for transferrin which has biantennary *N*-glycans (data not shown). So, β3Gn-T8 was speculated to have enzymatic specificity for some special structure of *N*-glycans. Substrate specificity for *N*-glycan is summarized in Table 2. The activity of β3Gn-T2 toward tetraantennary *N*-glycan without core fucose (Fucα1–6GlcNAc) is presented as 100% and all other activities are given as relative values. β3Gn-T8 only exhibited activity toward tetraantennary *N*-glycans, with or without core fucose. β3Gn-T8 yielded two products from tetra-antennary *N*-glycan. One had GlcNAc on a Gal residue of the β1–2 branch, and the other had GlcNAc on the β1–6 branch. β3Gn-T8 prefers the β1–2 branch to the β1–6 branch (data not shown). Ujita et al. [29] reported that i-extension enzyme (iGnT), which can synthesize polylactosamine chain on *N*-glycan, transfers GlcNAc to β1–2 branch more preferentially than β1–6 branch of *N*-glycan. Though both enzymes, β3Gn-T8 and iGnT, have a low homology, they show a similar acceptor specificity toward tetraantennary *N*-glycan.

3.3. Biosynthesis of polylactosamines in HCT15 cells

To determine the enzymatic activity of β3Gn-T8 in vivo, HCT15 cells were transfected with pcDNA3.1-β3Gn-T8 or an empty vector, and examined for changes in the cell surface expression of carbohydrate chains by flow cytometric analysis

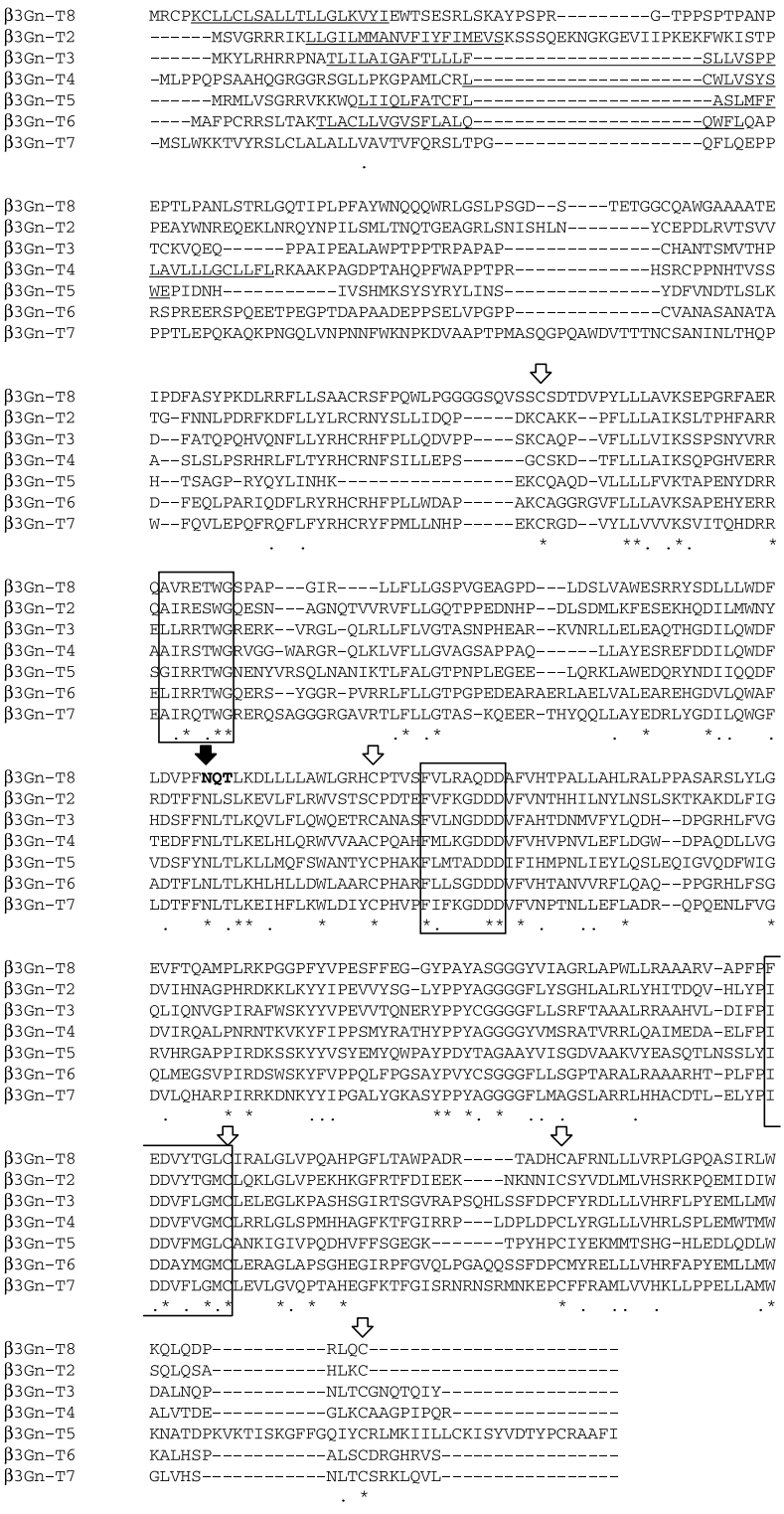

Fig. 1. ClustalW alignment for comparison of β3Gn-T8 with the other six β3Gn-Ts. Introduced gaps are shown with *hyphens*. The three β3-GT motifs are *boxed*. The putative transmembrane domains are *underlined*. *Asterisks* indicate the amino acids identical among all proteins. Conserved amino acids are shown by *dots*. Triplets of aspartic acid residues, DDD, are exited in the second β3-GT motif. Cysteine residues conserved in the seven β3Gn-Ts, β3Gn-T2, -T3, -T4, -T5, -T6, -T7, and -T8 are indicated by *open arrows*. Possible *N*-glycosylation sites in the β3Gn-T8 sequence are indicated by *bold letters*. A possible *N*-glycosylation site conserved in all proteins is indicated by a *closed arrow*.

using LEA (tomato) and PHA-L4 lectins, which recognize polylactosamine and β1,6-branched *N*-glycan, respectively. As shown in Fig. 4, the transfectants exhibited increased levels of both LEA and PHA-L4, compared with mock transfectants. This result indicated that β3Gn-T8 is involved in the biosynthesis of polylactosamine chains on the β1,6-branch of *N*-glycan. Considering that β3Gn-T8 only acts on tetraantennary *N*-glycans, β3Gn-T8 initiates the elongation of polylactos-

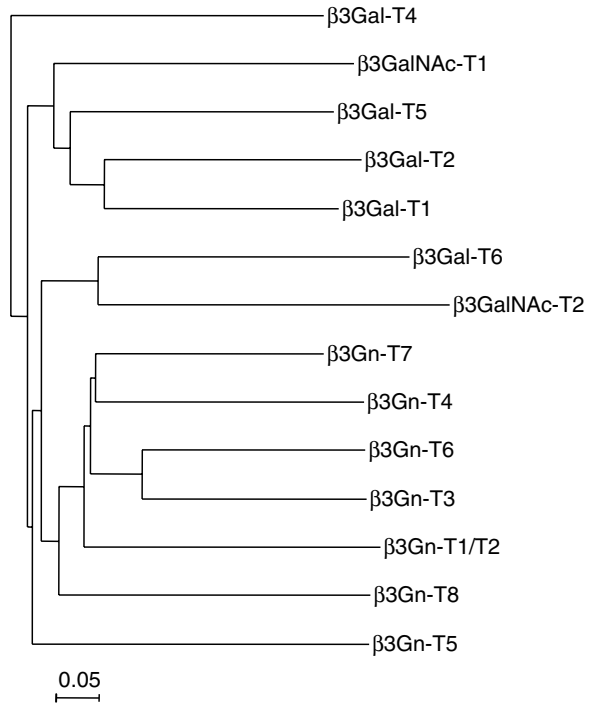

Fig. 2. A phylogenetic tree of human β3Gal-Ts, β3GalNAc-Ts and β3Gn-Ts. A phylogenetic tree of human β3-glycosyltransferases was constructed by means of the neighbor joining method based on the amino acid sequences. The branch length indicates the evolutionary distance between different sequences.

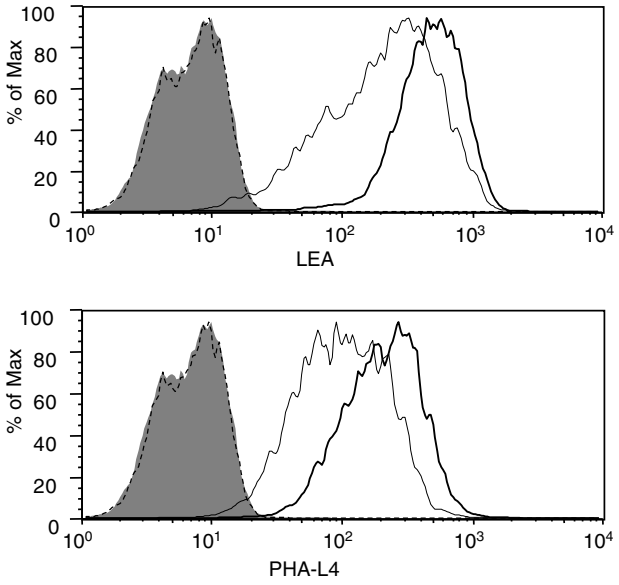

Fig. 4. Flow cytometric analysis of HCT15 cells transfected with the *β3Gn-T8* Gene. The expression of lactosamine and β1,6-branching *N*-glycans was recognized by LEA and PHA-L4 lectin, respectively. HCT15/β3Gn-T8 and HCT15/mock stained with and without lectin on the cell surface were analyzed by flow cytometry. HCT15/β3Gn-T8 cells are stained with lectin (*bold line*) or without lectin (*dotted line*) and HCT15/mock are stained with lectin (*thin line*) or without lectin (*shaded area*), respectively, in each panel.

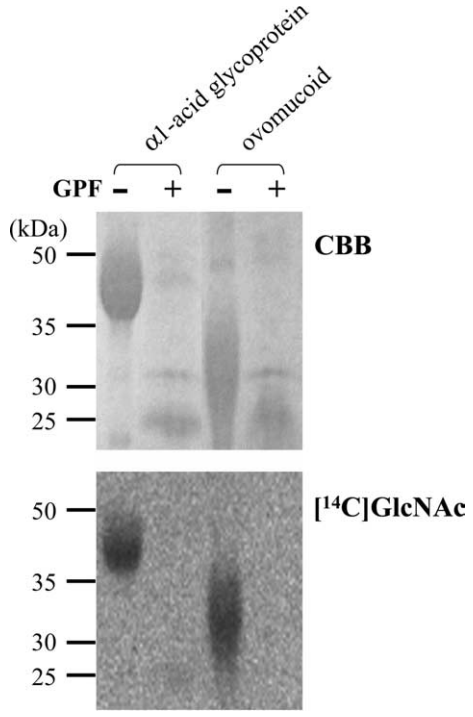

Fig. 3. Assay to determine the β3Gn-T8 activity toward α1-acid glycoprotein and ovomucoid. The reaction mixtures of enzyme and substrate were not treated (−) or treated (+) with Glycopeptidase F (GPF), and then separated by SDS–PAGE. The gels were stained with CBB (*upper panel*) or subjected to autoradiography (*lower panel*).

amine on the β1,2- and/or β1,6-branched structure of *N*-glycan, which is in accord with the structures found in nature.

3.4. Tissue distribution of the *β3Gn-T8* transcript

The level of expression of the *β3Gn-T8* transcript in various human tissues was determined by quantitative real-time PCR. As shown in Fig. 5, this gene was expressed in almost all tissues except the colon, although the level of expression varied with the tissue. Tissues expressing high levels of *β3Gn-T8* were small intestine, pancreas, spleen and bone marrow. Those expressing low levels were fetal brain, cerebellum, heart, liver and testis. The level in colon was almost undetectable.

The results of the analysis of clinical samples are summarized in Fig. 6. In most cases, the level of *β3Gn-T8* transcript was increased markedly in colon cancer tissues compared to normal tissue. In addition, most of the cell lines established from colon cancer showed higher levels of the *β3Gn-T8* transcript than did the cell lines derived from gastric cancer, esophageal cancer, hepatocellular carcinoma and pancreas cancer. Levels of transcript and activity of some glycosyltransferases are known to increase in cancerous tissue, for example, *N*-acetylglucosaminyltransferase V (GnT-V), fucosyltransferase IV, and ST3Gal II in colorectal cancer [30,31]. GnT-V catalyzes β1–6 branching of *N*-acetylglucosamine on *N*-glycan, and its expression is correlated with metastasis and poor prognosis in colorectal cancer [30]. The β1–6 branching structure, the product of GnT-V, is a good substrate for the attachment of polylactosamine [29]. In a study on colon carcinoma cell lines, highly metastatic cell lines were found to synthesize more *N*-glycans that contain polylactosamines than poorly metastatic cell lines [32]. The

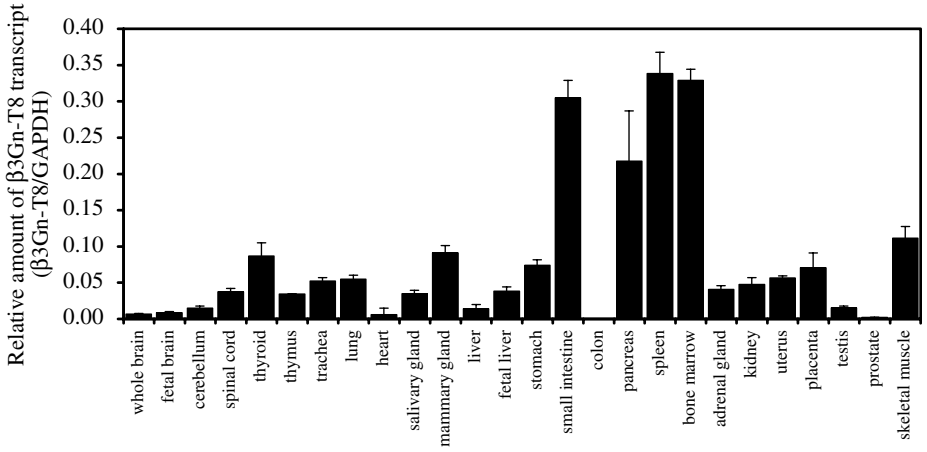

Fig. 5. Quantitative real-time PCR analysis of the *β3Gn-T8* transcript in various human tissues. Standard curves for β3Gn-T8 and GAPDH were generated by serial dilution of each plasmid DNA. The expression level of the *β3Gn-T8* transcript was normalized to that of the GAPDH transcript, which was measured in the same cDNAs. Data were obtained from triplicate experiments and are indicated as means ± S.D.

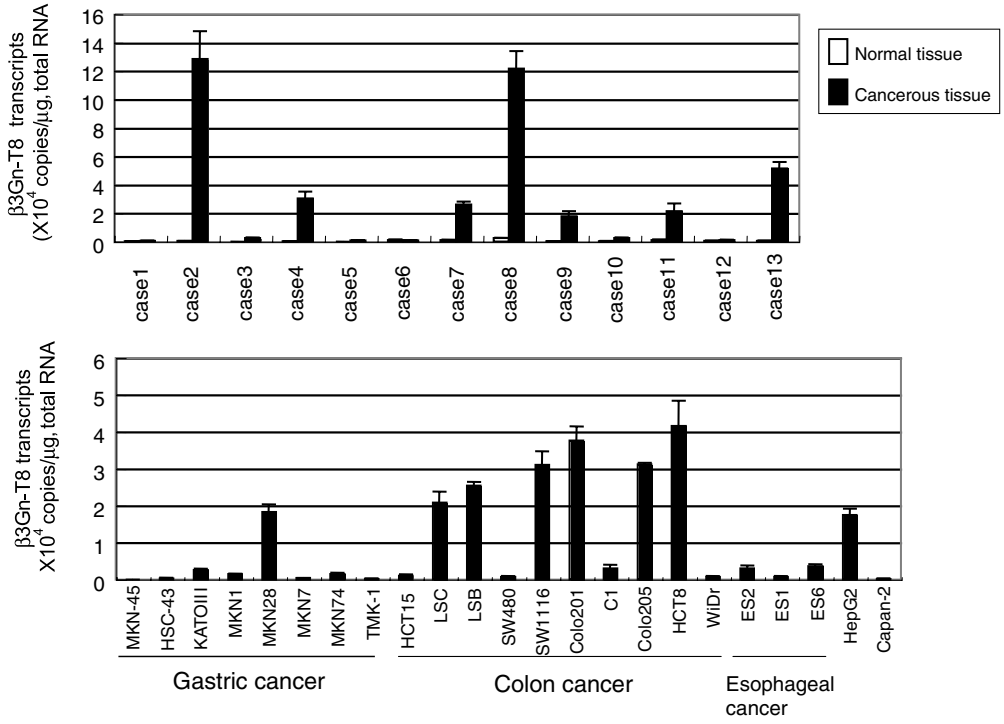

Fig. 6. Quantitative real-time PCR analysis of the *β3Gn-T8* transcript in normal, cancerous tissues and cell lines. The expression of β3Gn-T8 in normal (*open bar*) and cancerous (*closed bar*) tissues was assayed with colon tissues. A standard curve for β3Gn-T8 was generated by serial dilution of plasmid DNA. The relative amount of *β3Gn-T8* transcript was normalized to the amount of total RNA. Data were obtained from triplicate experiments and are indicated as means ± S.D.

terminus of the polylactosamine chain is preferentially fucosylated to form sialyl Lewis X. The expression level of sialyl Lewis X is also correlated with tumor recurrence and survival in colorectal cancer [33].

In this study, we found a novel member of the β3-GT family, β3Gn-T8, which can extend polylactosamine on *N*-glycan. Taking into consideration that the transcripts of *β3Gn-T8* are abundant in most colorectal cancer tissue, β3Gn-T8 may be involved in malignancy, by synthesizing polylactosamine on β1–6 branched *N*-glycans, in colon cancer.

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